Behavioral/Systems/Cognitive

Maternal Care and DNA Methylation of a Glutamic Acid Decarboxylase 1 Promoter in Rat Hippocampus

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Parenting and the early environment influence the risk for various psychopathologies. Studies in the rat suggest that variations in maternal care stably influence DNA methylation, gene expression, and neural function in the offspring. Maternal care affects neural development, including the GABAergic system, the function of which is linked to the pathophysiology of diseases including schizophrenia and depression. Postmortem studies of human schizophrenic brains have revealed decreased forebrain expression of glutamic acid decarboxylase 1 (*GAD1*) accompanied by increased methylation of a *GAD1* promoter. We examined whether maternal care affects *GAD1* promoter methylation in the hippocampus of adult male offspring of high and low pup licking/grooming (high-LG and low-LG) mothers. Compared with the offspring of low-LG mothers, those reared by high-LG dams showed enhanced hippocampal *GAD1* mRNA expression, decreased cytosine methylation, and increased histone 3–lysine 9 acetylation (H3K9ac) of the *GAD1* promoter. DNA methyltransferase 1 expression was significantly higher in the offspring of low- compared with high-LG mothers. Pup LG increases hippocampal serotonin (5-HT) and nerve growth factor-inducible factor A (NGFI-A) expression. Chromatin immunoprecipitation assays revealed enhanced NGFI-A association with and H3K9ac of the *GAD1* promoter in the hippocampus of high-LG pups after a nursing bout. Treatment of hippocampal neuronal cultures with either 5-HT or an NGFI-A expression plasmid significantly increased *GAD1* mRNA levels. The effect of 5-HT was blocked by a short interfering RNA targeting NGFI-A. These results suggest that maternal care influences the development of the GABA system by altering *GAD1* promoter methylation levels through the maternally induced activation of NGFI-A and its association with the *GAD1* promoter.

Introduction

Parenting influences endophenotypes that associate with a wide range of psychopathologies (Rapee, 1997; Carter et al., 2002). The challenge is that of establishing a direct relationship between parental care and the development of the neural systems that mediate the relevant cognitive and emotional endophenotypes, as well as identifying candidate mechanisms. One approach is that of examining maternal care in the rat, notably variations in pup licking/grooming (LG) (Liu et al., 1997; Francis et al., 1999; Weaver et al., 2004a,b). The adult offspring of mothers that naturally showing low levels of pup LG (i.e., low-LG mothers) show enhanced stress reactivity (Liu et al., 1997; Caldji et al., 1998; Francis et al., 1999; Weaver et al., 2004b; Toki et al., 2007), including increased dopamine stress responses in the medial prefrontal cortex (PFC) (Zhang et al., 2005). These animals also exhibit impaired sensorimotor gating as measured by prepulse inhibition (PPI) (Zhang et al., 2005). Impairments in PPI associate with an increased risk for psychopathology (Geyer et al., 1990, 2001). Manipulations of rearing conditions influence the development of systems that mediate PPI (Ellenbroek et al., 1998; Ellenbroek and Cools, 2000, 2002).

The forebrain expression of glutamic acid decarboxylase (GAD), the rate-limiting enzyme in GABA synthesis, is decreased in schizophrenic patients (Akbarian et al., 1995; Guidotti et al., 2000b; Volk et al., 2000; Heckers et al., 2002). Such effects are a potential mechanism for the altered dopamine function that underlies psychosis (Benes, 1997). Studies with postmortem brain samples from schizophrenic patients reveal increased DNA methylation of the *GAD1* promoter (Costa et al., 2006; Grayson et al., 2006; Kundakovic et al., 2007). DNA methylation associates with gene silencing and cortical GABAergic neurons from schizophrenic patients also show increased expression of DNA methyltransferase 1 (Ruzicka et al., 2007), which catalyzes cytosine methylation (Buryanov and Shevchuk, 2005; Goll and Bestor, 2005). Decreased *GAD1* transcription is linked to increased DNMT1 association with the *GAD1* promoter (Kundakovic et al., 2007). However, the developmental origins of such differences in the transcriptional regulation of *GAD1* are unknown.

Maternal care alters hippocampal glucocorticoid receptor (GR) gene expression in the adult offspring through effects on DNA methylation of a neuron-specific, exon 1_7 GR promoter. The maternal effect is mediated by enhanced serotonergic activity and increased expression of nerve growth factor-inducible factor A (NGFI-A) (Weaver et al., 2007). Thus, there is increased NGFI-A association with the exon 1_7 promoter in pups of high-

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compared with low-LG mothers (Weaver et al., 2007). Serotonin (5-HT) induces demethylation of the exon 1_7 promoter and increases GR expression in cultured hippocampal neurons: Both effects are blocked by an antisense targeting NGFI-A (Weaver et al., 2007), whereas an NGFI-A expression plasmid mimics the 5-HT effect (Weaver et al., 2007). Interestingly, the *GAD1* promoter contains an NGFI-A consensus sequence (GenBank accession number AF110132.1), suggesting the intriguing possibility that the *GAD1* promoter may be similarly regulated by maternal care. The studies reported here provide evidence for such a maternal effect on the epigenetic regulation of *GAD1* expression that is comparable with that observed for the GR gene.

Materials and Methods

Animals

All procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care with protocols approved by the McGill University Animal Care Committee. The animals used were the male offspring of Long–Evans dams mated in our animal colony, which avoids confounds resulting from the stress of transportation. After mating, the females were housed singly on a standard 12 h light/dark cycle (lights on at 8:00 A.M.) in polycarbonate maternity cages containing bedding, with *ad libitum* access to food and water. Cage cleaning began no earlier than postnatal day 10. Litters were left otherwise undisturbed with their respective dams until weaning on day 21 after which time the offspring from each litter were housed together in same-sex groups of three to four per cage until postnatal day 45 (PND45). From PND45 onward, animals from each litter were housed in pairs until testing, which occurred at 3–4 months of age. One or two animals per litter were used for the study.

Assessment of maternal behavior

Maternal behavior was assessed using a procedure adapted from that previously described (Liu et al., 1997; Champagne et al., 2003). The frequency of maternal behaviors was scored on postpartum days 1 through 6. Observers were trained to a high level of interrater reliability (>0.90). Dams were observed in their home cage and not disturbed for the duration of the 6 d observation period. Daily observations occurred during five, 75 min sessions, three of which were scheduled during the light phase (10:00 A.M., 1:00 P.M., and 5:00 P.M.) and two during the dark phase (7:00 A.M. and 8:00 P.M.) of the light cycle. Within each observation session, the behavior of each mother was scored 25 times (one observation/3 min) for pup LG (including both body and anogenital licking). Thus, each mother's LG frequency score was based on a total of 750 observations (25 observations/session × 5 session/d × 6 d = 750 observations/mother) and was expressed as percentage occurrence (number of occurrences/750 × 100).

Pups were designated as high- or low-LG on the basis of their mother's LG frequency score relative to the mean ± 1 SD frequency score for the cohort (~60–80 mothers/cohort). High-LG mothers were defined as females whose LG frequency scores were >1 SD above the cohort mean. Low-LG mothers were defined as females whose LG frequency scores were >1 SD below the cohort mean.

Quantitative real-time PCR

Whole brains were rapidly removed and stored at −80°C. The hippocampal tissue was dissected and RNA extracted from one hemisphere using RNeasy mini kit (QIAGEN; catalog #74106) with on-column DNase digestion (QIAGEN; catalog #79254). The overall quality and yield of the RNA preparation was determined using SmartSpec plus spectrophotometer (Bio-Rad Laboratories). cDNA synthesis was completed using reverse transcriptase AMV (Roche Applied Science; catalog #10109118001). Quantitative real-time PCR was performed with a LightCycler 480 (Roche Applied Science) and RT2 PCR primer set for rat *GAD1* (Superarray Bioscience Corporation; catalog #PPR06836A). β2-Microglobulin (β2M) (Superarray Bioscience Corporation; catalog #PPR42607A) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Superarray Bioscience Corporation; catalog #PPR06557A) reference genes from same sample were amplified to control for potential loading errors. The results were identical with both controls and the results are expressed relative to β2M.

Chromatin immunoprecipitation assay

Animals were perfused with 4% paraformaldehyde to cross-link protein–DNA complexes (Weaver et al., 2004b) and then stored at −80°C until dissection. Hippocampi were dissected and chromatin was immunoprecipitated using rabbit polyclonal antibody to NGFI-A (Santa Cruz Biotechnology), acetylated histone H3–lysine 9 (Millipore; catalog #07-352), or normal rabbit IgG nonimmune antibody (from Santa Cruz Biotechnology). One-tenth of the lysate was kept before immunoprecipitation and used to quantify DNA levels (input). The rat hippocampal *GAD1* promoter region (GenBank accession number NM_017007.1; assay position, −399; chromosome ref sequence #NC_005102.2; TSS position, 52789370) of the un-cross-linked DNA was subjected to quantitative PCR (qPCR) amplification [primer set; Superarray Bioscience Corporation; catalog #GPR060837(−)01A].

Sodium bisulfite mapping

Cytosine methylation was determined using sodium bisulfite conversion followed by sequencing (Frommer et al., 1992; Clark et al., 1994). Hippocampal genomic DNA (0.5 μg) was treated with sodium bisulfite (EpiTect Bisulfite kit; QIAGEN; catalog #59104). The *GAD1* promoter region (GenBank accession number AF110132) of bisulfite-treated DNA (0.5 μg) was PCR amplified using outside primers (forward, 589-taa aaa gga aga gaa aga aga att att; reverse, 1074-tta aac tta act tta ctc tac cac aaa c). The PCR protocol included an initial denaturating cycle (5 min, 95°C), followed by 34 cycles of denaturation (1 min, 95°C), annealing (2 min 30 s, 55°C), and extension (1 min, 72°C), followed by a final extension cycle (7 min, 72°C) terminating at 4°C. The PCR product was used as a template for subsequent PCR amplification using nested primers (forward, 828-ata ttt gta aag gag ttt tag gtt g; reverse, 1016-tcc tta ctt aca aaa tcc cta atc c). The nested PCR product was cleaned using QIAquick PCR purification kit (QIAGEN; catalog #28104), subcloned (QIAGEN PCR cloning kit; catalog #231124), and transformed to produce 20 different colonies per plate. Twenty plasmids per animal containing the ligated *GAD1* promoter DNA fragment were sequenced at the Genome Québec Innovation Centre (McGill University, Montreal, Quebec, Canada).

Hippocampal cell culture

Hippocampi were dissected from embryonic day 20 embryos, trypsinized, homogenized, and seeded onto 60 mm plates at a density of ~3×10^7 cells per plate. Media consisted of MEMα (Invitrogen) supplemented with 10% FBS, 20 mM KCl, 0.25% glucose, 15 mM HEPES, 0.1% penicillin/streptomycin, and 20 μM 5′-fluorodeoxyuridine to prevent glial cell growth. Media was changed the following day and contained lentiviral vectors containing a short hairpin RNA (shRNA) directed against NGFI-A, an overexpression vector (OE) containing NGFI-A cDNA (Changelian et al., 1989), or the appropriate empty vector (EV) as control. In the overexpression experiments, the NGFI-A overexpression vector or empty vector were added the day after plating and the medium was changed every 3–4 d. In the shRNA experiments, cells were treated with a lentiviral vector containing NGFI-A shRNA or no insert (empty vector) the day after plating. After 3 d of lentiviral treatment, the medium was removed and replaced with either control media or media containing 1 μM serotonin. Cells were harvested 9 d after plating in PBS and stored at −80°C. RNA was isolated using Roche HiPure RNA extraction kit. Reverse transcription reactions were then performed on 0.5 μg of RNA using Roche AMV reverse transcriptase. cDNA thus generated was analyzed using quantitative real-time PCR (qRT-PCR). GAD67 protein level was measured by Western blot after 4 d of serotonin treatment (9 d after plating) in a separate experiment.

Production of recombinant lentiviral vectors

Plasmid construction. Viral vectors were derived from the human immunodeficiency virus-based lentiviral backbones generously supplied by collaborators (S. D. Andrews and M. Szyf, McGill University, Montreal, Quebec, Canada). For overexpression vector, NGFI-A cDNA (Changelian et al., 1989) was ligated into the pLenti6/V5-Topo vector plasmid

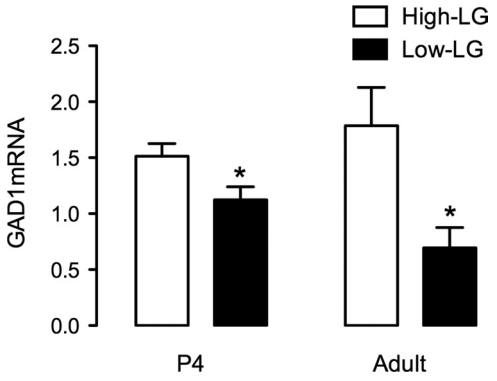

Figure 1. Influence of maternal care on hippocampal *GAD1* expression. Mean ± SEM levels of GAD1 mRNA quantified using qRT-PCR analysis of the amplified GAD1 mRNA (relative to the β2M standard) in the postnatal day 4 and adult offspring of high- or low-LG mothers (n = 4–8/group; *$p < 0.05$).

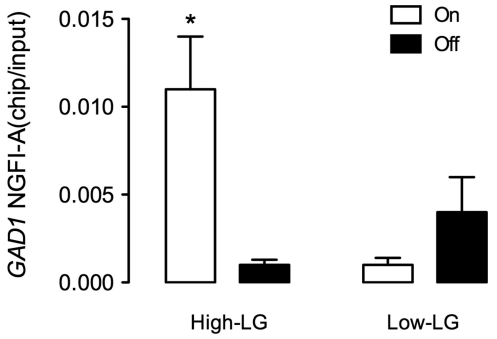

Figure 2. Maternal regulation of NGFI-A binding to the GAD1 promoter. Mean ± SEM qPCR data of the amplified GAD1 promoter sequence from NGFI-A immunoprecipitated hippocampal extract as a ratio of that from the input fraction. Hippocampal samples were obtained from the male, postnatal day 4 pups of high- or low-LG mothers immediately after a nursing bout (ON) or 25 min after the termination of a nursing bout (without any subsequent mother–pup interaction; OFF; n = 4–6/group; *$p < 0.05$ from all other groups).

(Invitrogen). The resulting expression plasmid contains a cytomegalovirus (CMV) promoter driving expression of NGFI-A or a random sequence (empty vector). For the short interfering RNA (siRNA)-containing vector, NGFI-A siRNA was acquired commercially (Open Biosystems) and subcloned into a pLVTHM plasmid (TronoLab) along with a GFP (green fluorescent protein) expression tag.

Preparation of virus. Virus was generated by transient cotransfection of the expression plasmid (15 μg), envelope plasmid (pMD2.G; TronoLab; 5 μg), and the packaging plasmids [for overexpression, 10 μg of third generation packaging plasmids pRSVrev and pMDLg pRRE (TronoLab) were used; for siRNA, 10 μg of second-generation packaging plasmid psPAX2 (TronoLab)] into a 150 mm plate of 90% confluent HEK293T by calcium phosphate precipitation. Medium was collected 48 and 72 h after transfection, cleared of debris by low-speed centrifugation, and filtered through 0.45 μm filters. High-titer stocks were prepared by an initial ultracentrifugation for 1 h at 138,000 × *g*. Viral pellet was resuspended in sterile PBS and stored at 80°C. After concentration typical titers ranged from 10^7–10^8 TU/ml. Sufficient virus was added to cultures to provide multiplicities of infection of 10.

Statistical analysis

All statistical analyses were conducted using SPSS 15.0. Simple comparisons between two groups (high-LG and low-LG) were analyzed by independent Student's t tests. For analyses involving factorial designs (maternal care and licking bout condition), the primary analysis conducted was a two-way ANOVA. Significant main effects and interactions were interpreted using Tukey's *post hoc* tests.

Results

Hippocampal GAD1 expression

We first examined differences in hippocampal GAD1 mRNA expression in the offspring of high- and low-LG mothers using qRT-PCR (with β2M as reference gene). There was a significantly ($t = 2.82$; df = 6; $p = 0.03$) higher level of GAD1 mRNA in the adult offspring of high- compared with low-LG dams. This same maternal effect ($t = 2.40$; df = 14; $p = 0.03$) was also apparent at postnatal day 4 (Fig. 1), suggesting dynamic regulation of *GAD1* expression during early postnatal development.

Maternal regulation of NGFI-A association with GAD1 promoter

Maternal effects on hippocampal gene expression are associated with an increase in NGFI-A expression and NGFI-A association with relevant promoter sequences (Weaver et al., 2007). We used chromatin immunoprecipitation (ChIP) assays to examine NGFI-A association with the *GAD1* promoter in postnatal day 4 pups as a function of concurrent mother–pup interaction. Hippocampal samples were obtained from pups immediately after periods of interaction (an "on" period) or a period in which the mother had been away from the litter for a minimum of 25 min without physical contact between mother and pup (i.e., an "off" period). An "on" bout was operationally defined as a period of observations in which the mother was in constant contact with the pups and recorded as being engaged in pup LG behavior. Note previous studies (Champagne et al., 2003, 2004) show that the differences in the pup LG between high- and low-LG mothers derive from a difference in the duration, but not the frequency of pup LG. In this study, the duration of an LG bout was 9.9 ± 1.5 min in the high-LG mothers and 3.1 ± 0.7 min in low-LG mothers.

The results (Fig. 2) showed a significant interaction between the effects of maternal care and nursing bout condition ($F_{(1,14)} = 13.62$; $p = 0.002$). *Post hoc* tests confirmed that the association of NGFI-A with the *GAD1* promoter was significantly ($p < 0.05$) increased in pups of high- compared with low-LG mothers, but only after an "on" period ($p = 0.005$). Samples obtained 25 min after no interaction between the mother and pup (i.e., the "off" period) showed no maternal effect on NGFI-A association with the *GAD1* promoter (low-LG on vs low-LG off, $p = 0.68$). These findings show an increase in NGFI-A association with the *GAD1* promoter at the same period in life in which differences in *GAD1* expression are apparent (Fig. 1).

In vitro regulation of *GAD1* expression

The effects of maternal care on hippocampal gene expression are associated with an increase in 5-HT hippocampal turnover and increased NGFI-A expression (Mitchell et al., 1992; Meaney et al., 2000; Weaver et al., 2001, 2007). We found that 5-HT treatment of primary hippocampal neuronal cell cultures significantly increased GAD_{67} expression in hippocampal cultures (Fig. 3*a*,*b*) ($t = 4.77$; df = 7; $p = 0.002$). Likewise, infection of primary hippocampal neuronal cultures with a lentivirus bearing an NGFI-A expression plasmid (Fig. 3*c*) was associated with a significant ($F_{(2,8)} = 5.66$; $p = 0.03$) increase in GAD1 mRNA expression. *Post hoc* analysis showed that GAD1 mRNA levels were significantly ($p < 0.01$) higher than those in either the no-treatment or empty vector controls groups (Fig. 3*d*).

We then examined whether the effect of 5-HT on hippocampal GAD1 expression was mediated through NGFI-A. Hippocampal cultures were treated with 5-HT together with a lentiviral construct bearing a siRNA against NGFI-A mRNA or

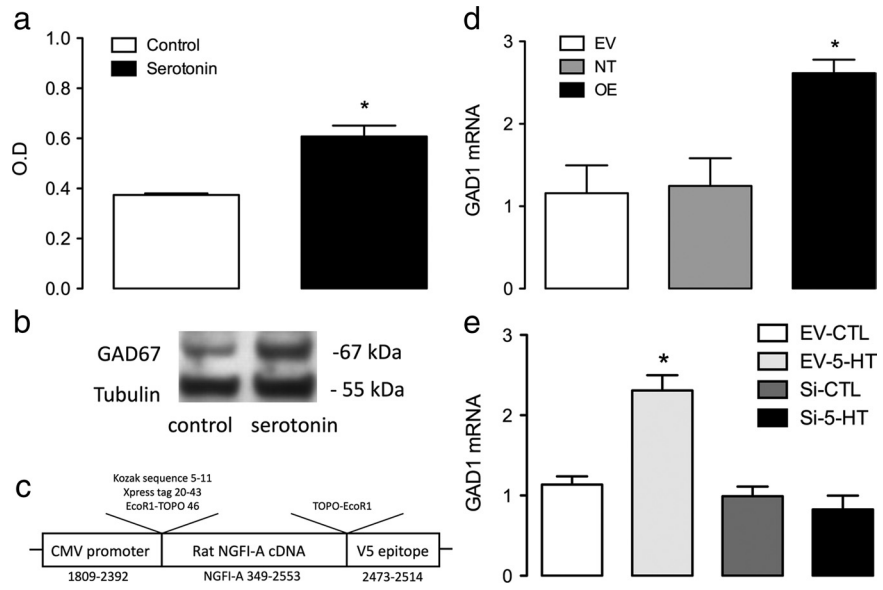

Figure 3. Regulation of GAD1 expression in primary hippocampal neuronal cell cultures. ***a***, Mean ± SEM levels of GAD_{67} expression (relative optical density) quantified using Western blotting with samples from hippocampal cultures treated with serotonin (5-HT) (1 μM) or medium alone (*$p < 0.01$). ***b***, Representative Western blots showing bands corresponding to GAD_{67} and α-tubulin (used to control for loading variation). ***c***, Physical map of the CMV/V5-His-NGFI-A expression vector. ***d***, Mean ± SEM levels of GAD1 mRNA quantified using qRT-PCR analysis of the amplified GAD1 mRNA (relative to the β2M standard) in primary hippocampal neuronal cultures treated with a lentiviral vector containing an NGFI-A expression plasmid (OE), the empty viral vector (EV), and a reference, no-treatment group (NT) ($n = 8$/group; *$p < 0.05$). ***e***, Mean ± SEM levels of GAD1 mRNA amplified from primary hippocampal neuronal cultures, quantified using qRT-PCR (with β2M as standard), treated with medium alone plus empty viral vector (EV–CTL), 5-HT in medium plus empty viral vector (EV–5-HT), medium alone plus the viral vector containing an NGFI-A siRNA (SI–CTL), and 5-HT in medium plus the viral vector containing an NGFI-A siRNA (SI–5-HT; $n = 4–6$/group) in sample (*$p < 0.01$ compared with all other groups).

an empty viral construct. There was a significant treatment effect ($F_{(1,22)} = 8.18$; $p = 0.01$). *Post hoc* analysis revealed significantly ($p < 0.05$) lower GAD1 mRNA levels in cells cotreated with either 5-HT or media alone as well as with the NGFI-A siRNA compared with cells treated with 5-HT and the empty vector control. Among cultures treated with the empty vector, there was also a significant increase in GAD1 mRNA expression in 5-HT-treated compared with media alone (Fig. 3*e*). The results suggest that 5-HT treatment increases GAD1 expression and that this effect is mediated by NGFI-A.

GAD1 promoter methylation

Pup LG increases NGFI-A association with a *GAD1* promoter. Increased NGFI-A binding in postnatal development is associated with alterations in chromatin structure and DNA methylation (Weaver et al., 2007). We used sodium bisulfite mapping in the region around the NGFI-A consensus sequence sites (Fig. 4*a*) to determine whether maternal care affects DNA methylation of the *GAD1* promoter in adult hippocampus and prefrontal cortex. Since incomplete bisulfite conversion of cytosines will complicate sequence analysis, only sequences containing less than two unconverted sites in non-CpG positions were chosen for analysis. Our conversion efficiency in the current study was 99.8% in non-CpG sites, which compares favorably with that commonly reported in the literature (Kumaki et al., 2008).

Consistent with reports from other promoter regions (Bird, 1986), cytosine methylation levels were generally low across the *GAD1* promoter. However, there were significant differences in methylation levels in hippocampal samples from the adult offspring of high- and low-LG mothers (Fig. 4*b*,*c*). We compared the percentage of methylated clones in each group. Clones with one or more methylated sites were counted as "methylated," and this value was divided by the total number of clones. The percentage of methylation in hippocampal samples was significantly higher in low- compared with high-LG (Fig. 4*b*) (30.0 vs 8.7%; $t = -3.91$, df = 8, $p = 0.004$). We did not observe site-specific differences in methylation (Fig. 4*c*). However, CpG dinucleotides at sites 1–4 or sites 11–13 were among those sites most likely to show methylation, and the latter lie within the NGFI-A consensus sequence (Fig. 4*a*). Moreover, we did not find significant differences in methylation levels in the prefrontal cortex (mean percentage ± SEM: high-LG, 13.4 ± 4.0, vs low-LG, 32.7 ± 17.3; $p = 0.3$).

Hippocampal DNMT1 expression

DNMT1 plays an important role in the regulation and maintenance of DNA methylation, including the *GAD1* promoter (Kundakovic et al., 2007, 2009). Thus, we examined differences in hippocampal DNMT1 mRNA expression in the offspring of high- and low-LG mothers using qRT-PCR (with β2M as reference gene). We found that DNMT1 expression was significantly higher in the offspring of low- compared with high-LG mothers (Fig. 5*a*) ($t = -2.50$; df = 8; $p = 0.04$).

Histone H3 lysine-9 acetylation of the GAD1 promoter

DNA methylation is associated with alteration in chromatin states and transcription factor binding. Such effects can be mediated through effects on histone acetylation. The *GAD1* promoter showed an approximately twofold and statistically significant ($t = 2.45$; df = 10; $p = 0.03$) (Fig. 5*b*,*c*) increase in H3-K9 acetylation in samples from the offspring of high- compared with low-LG mothers, as measured using ChIP with an antibody against acetylated H3-K9. H3-K9ac accompanies open chromatin states, increased transcription factor binding and active gene transcription. Thus, we then examined NGFI-A association with the *GAD1* promoter in samples from the adult offspring of high- and low-LG mothers using ChIP assays (Fig. 5*b*). There was significantly ($t = 2.64$; df = 7; $p = 0.03$) increased levels of NGFI-A association with the *GAD1* promoter in the hippocampal samples from the adult offspring of high- compared with low-LG mothers. Note that we have previously shown that there are no differences in the levels of NGFI-A expression in the hippocampus of adult offspring of high- and low-LG mothers (Weaver et al., 2007). Thus, the differences in NGFI-A association with the *GAD1* promoter occur despite comparable levels of NGFI-A protein and suggest an epigenetically regulated level of transcription factor binding.

Discussion

Previous studies reveal an effect of variations in maternal care on the development of corticolimbic GABAergic systems (Caldji et al., 1998, 2000, 2003; Fries et al., 2004). Cross-fostering studies show that such alterations are directly related to postnatal mother–pup interactions (Caldji et al., 2003). The present study reveals a

maternal effect on *GAD1* expression in the hippocampus. This effect is tissue specific, with no comparable effect observed in the PFC.

Increased pup LG in the rat associates with enhanced binding of NGFI-A to the exon 1_7 GR promoter and to alterations in CpG methylation (Weaver et al., 2004b, 2007). In adults, the decreased CpG methylation of the exon 1_7 GR promoter increases H3-K9ac of and NGFI-A binding to the promoter, and increases GR expression. The differences in CpG methylation of the exon 1_7 promoter are reversed with cross-fostering between high- and low-LG mothers shortly after birth (Weaver et al., 2004b). The effect of pup LG is linked to an increase in hippocampal 5-HT turnover, and hippocampal cells treated with 5-HT show both a decrease in CpG methylation and an increase in GR expression; both effects are blocked with an siRNA directed against NGFI-A (Weaver et al., 2007). Likewise, NGFI-A overexpression leads to the demethylation of a methylated promoter construct. Site-directed mutagenesis within the NGFI-A response element of the construct eliminates NGFI-A binding and the effect on CpG methylation, suggesting that the binding of NGFI-A is essential for the effect on CpG methylation (Weaver et al., 2007).

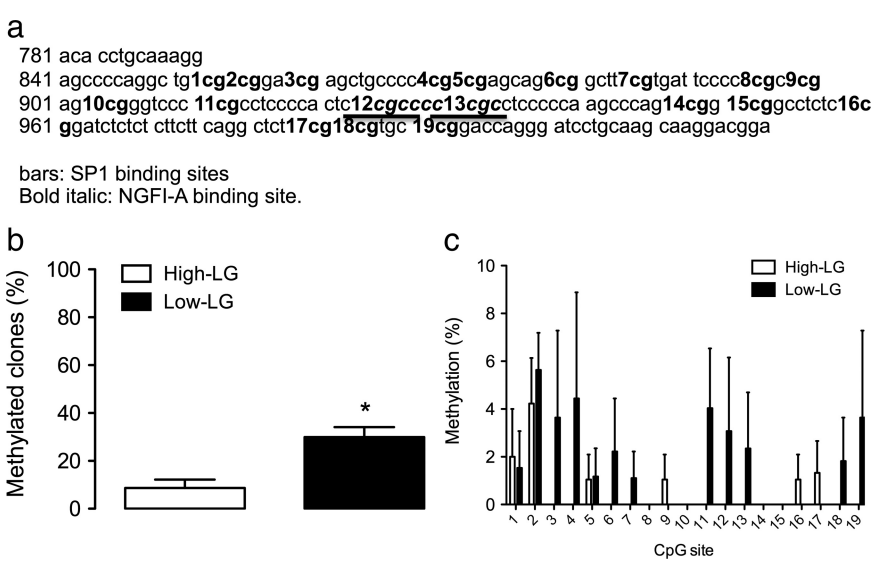

Figure 4. Maternal effect on GAD1 promoter methylation. ***a***, A sequence map of the GAD1 promoter showing the NGFI-A consensus sequence. ***b***, Mean ± SEM percentage of cloned GAD1 promoter sequences revealing one or more methylated CpG sites as determined using NaBis mapping (10–20 clones per sample, with $n = 5$/group; $^*p < 0.01$). ***c***, Mean ± SEM percentage of cloned GAD1 promoter sequences revealing one or more methylated CpG sites as determined using NaBis mapping for each of the individual CpG dinucleotides within the GAD1 promoter region. Statistical analysis reveals an overall group effect.

Studies of maternal programming of GR expression suggest that the interaction between NGFI-A and its consensus sequence might alter DNA methylation; thus, our interest in whether other NGFI-A genomic targets might be subject to regulation by variations in maternal care.The results of the present experiments suggest a pattern of findings comparable with those reported with the GR gene. Increased pup LG enhanced NGFI-A association with the *GAD1* promoter, and this effect was dependent on mother–pup interactions (Fig. 2). Likewise, 5-HT induced an increase in *GAD1* expression that was blocked with a siRNA to NGFI-A. The effect of 5-HT on *GAD1* expression in hippocampal neuronal cultures was mimicked by NGFI-A overexpression. This finding is consistent with that of a report showing that SDF1α-induced increases in *GAD1* expression in cultured hippocampal neurons are mediated by an increased association of NGFI-A to the *GAD1* promoter (Luo et al., 2008). These findings replicate previous results showing that maternally induced increases in NGFI-A expression are associated with an increase in NGFI-A association with NGFI-A-sensitive promoters, and stable epigenetic alterations (Weaver et al., 2004b, 2007). Indeed, Weaver et al. (2007) showed that an antisense oligonucleotide directed against NGFI-A could block the effects of 5-HT or cAMP on DNA methylation of the exon 1_7 GR promoter.

The degree to which DNA methylation states vary in postmitotic cells has only recently been fully appreciated (Meaney and Szyf, 2005; Sweatt, 2009). This "epigenetic plasticity" is of profound importance for neurons, which are a highly stable, yet phenotypically dynamic cell population. The mechanism driving such alterations in CpG methylation has yet to be clearly explained; however, the present studies together with those of GR gene promoter suggest that increased transcription factor binding might promote the remodeling of DNA methylation. Stat3 binding to the *gfap* promoter is implicated in the alterations to

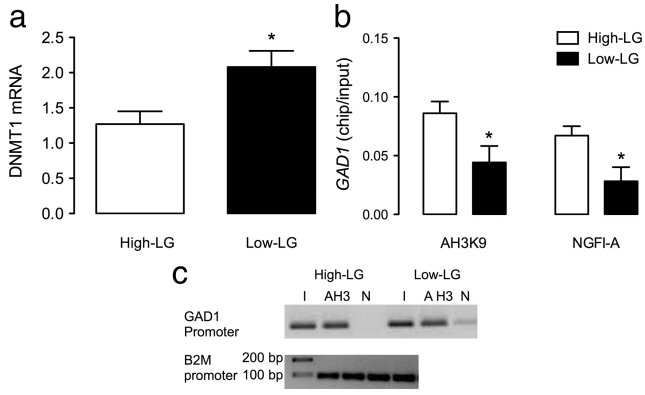

Figure 5. Maternal regulation of the epigenetic state of the GAD1 promoter. ***a***, Mean ± SEM levels of DNMT1 mRNA quantified using qRT-PCR analysis of the amplified DNMT1 mRNA (relative to the β2M standard) in hippocampal samples derived from adult offspring of high- or low-LG mothers ($n = 5$/group; $^*p < 0.05$). ***b***, Mean ± SEM qPCR data of the amplified GAD1 promoter sequence immunoprecipitated from hippocampal samples with an antibody to H3-K9ac or NGFI-A and normalized to the input values ($^*p < 0.05$; $n = 4$ samples/group). ***c***, Representative images of GAD1 and β2M qPCR products on agarose gel confirming the amplification of a single specific product from chromatin immunoprecipitation assays described above.

CpG methylation that accompany the increased *gfap* expression during gliogenesis (Namihira et al., 2004; Fan et al., 2005). SP-1 binding, even in the absence of transcriptional activation, results in the remodeling of DNA methylation (Brandeis et al., 1994; MacLeod et al., 1994). Thus, environmental regulation of protein–DNA interactions appear to mediate epigenetic plasticity at the level of DNA methylation (Martinowich et al., 2003; Meaney and Szyf, 2005; Weaver et al., 2007).

The altered *GAD1* expression in schizophrenia is associated with variations in DNA methylation (Costa et al., 2007). There is decreased *GAD1* expression in cortical neurons of schizophrenic patients compared with controls (Veldic et al., 2005; Ruzicka et al., 2007) that is accompanied by increased DNMT1 expression (Veldic et al., 2004; Ruzicka et al., 2007) and increased CpG

methylation (Costa et al., 2006; Mill et al., 2008). Such effects correspond to the decreased cortical GABAergic transmission and cognitive impairments in schizophrenia (Akbarian et al., 1995; Guidotti et al., 2000a; Lewis and Lieberman, 2000; Torrey et al., 2005). The downregulation of GAD1 mRNA is not associated with a change in the overall number of GABAergic neurons, suggesting an effect at the level of transcriptional regulation (Akbarian et al., 1995).

The adult offspring of low-LG mothers showed decreased hippocampal *GAD1* expression, increased CpG methylation of the *GAD1* promoter, and increased levels of DNMT1 mRNA. Grayson and colleagues (Kundakovic et al., 2007, 2009) provide evidence for a dynamic role of DNMT1 in the regulation of the DNA methylation state of the *GAD1* promoter. DNMT1 inhibition upregulates GAD1 expression (Kundakovic et al., 2007) and decreases methylation of the *GAD1* promoter (Kundakovic et al., 2009). Likewise, methionine treatment, which increases levels of the endogenous methyl donor *S*-adenosyl-methionine and thus CpG methylation, decreases GAD1 expression (Dong et al., 2005); this effect is blocked by a DNMT1 antisense (Noh et al., 2005). Interestingly, L-methionine treatment of schizophrenic patients worsens psychotic symptoms. These findings suggest a dynamic role for DNMT1 in the regulation of DNA methylation levels at selected genomic targets (Fan et al., 2005). DNMT1 acts in early development to maintain methylation patterns during cell replication. Interestingly, the adult expression of DNMT1 is pronounced in neurons, which are a stable cellular population at the level of replication, but remarkably dynamic at the level of function. These findings suggest that neuronal DNMT1 expression in adults is actively engaged in regulating methylation patterns. Moreover, DNMT1 expression in neurons is dynamically regulated by intracellular signaling pathways (Levenson et al., 2006). The same GABAergic neurons in the schizophrenic brain that express reelin and GAD_{67} exhibit an increase in DNMT1 (Veldic et al., 2004). Inhibition of DNMT1 in neuronal cell lines resulted in the increased expression of both reelin and GAD_{67} (Kundakovic et al., 2007).

The decreased hippocampal DNMT1 expression in the adult offspring of high-LG mothers was associated with decreased levels of DNA methylation across the *GAD1* promoter and an increased level of H3-K9ac of and NGFI-A binding to the *GAD1* promoter. The latter effect is observed despite the fact that the adult offspring of high- and low-LG mothers do not differ in constitutive levels of hippocampal NGFI-A expression (Weaver et al., 2007). H3-K9ac is a histone mark associated with open chromatin and increased transcription factor binding (Kadonaga, 1998; Roth et al., 2001). Increased cytosine methylation reduces levels of H3-K9ac and thus transcription factor binding through the recruitment of a repressor complex that commonly includes histone deacetylases (Klose and Bird, 2006). We suggest that the increased level of DNA methylation across the *GAD1* promoter reduces NGFI-A binding and GAD1 transcription. In the case of hippocampal GR expression, chronic infusion of a histone deacetylase (HDAC) inhibitor into the adult offspring of low-LG mothers increases H3-K9ac of the exon 1_7 GR promoter, NGFI-A association with its consensus sequence, and GR expression (Weaver et al., 2004b). The effects are accompanied by a decreased level of cytosine methylation of the exon 1_7 promoter. HDAC inhibitors exert a comparable effect on GAD1 expression *in vivo* (Tremolizzo et al., 2002).

Overall levels of DNA methylation across the *GAD1* promoter were generally low, raising the obvious question of whether such differences are indeed functionally relevant. The transcriptional silencing associated with methylation is mediated, in part, by the binding of methylated DNA binding proteins (MDBs) and the recruitment of repressor complexes (Klose et al., 2005; Klose and Bird, 2006). The binding of MDBs to DNA is remarkably sensitive requiring as few as one to two methylcytosines (Nan et al., 1993). These findings suggest that even low levels of methylation could potentially result in the recruitment of MDBs to the DNA site and alterations in transcriptional activity. Importantly, over three times as many *GAD* promoter clones were methylated at at least one CpG site in samples from adult offspring of low- compared with high-LG mothers (Fig. 4*b*).

These studies suggest that variations in maternal care can regulate the epigenetic state and expression of genes implicated in psychopathology, suggesting a specific developmental origin for the epigenetic modifications associated with mental health disorders (Mill et al., 2008). McGowan et al. (2009) provide evidence for a link between the quality of parent–child interactions and the methylation state and expression of the GR gene in human hippocampus. The findings presented here suggest that maternal care influences the development of the GABA system through epigenetic effects on selected genomic targets. The results are consistent with the idea that alterations in DNA methylation underlie the environmental regulation of genotype–phenotype relationships that influence neural function and mental health.

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